

Alternaria alternata and Its Allergens: a Comprehensive Review

Irena Kustrzeba-Wójcicka · Emilia Siwak ·
Grzegorz Terlecki · Anna Wolańczyk-Mędrala ·
Wojciech Mędrala

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Abstract *Alternaria alternata* is mainly an outdoor fungus whose spores disseminate in warm, dry air, so in temperate climates, their count peaks in the summers. *Alternaria* may also be found in damp, insufficiently ventilated houses, where its allergenic properties cocreate the sick building syndrome. Mold-induced respiratory allergies and research on *Alternaria* both have a lengthy history: the first was described as early as 1698 and the second dates back to 1817. However, the two were only linked in 1930 when *Alternaria* spores were found to cause allergic asthma. The allergenic extracts from *Alternaria* hyphae and spores still remain in use but are variable and insufficiently standardized as they are often a random mixture of allergenic ingredients and coincidental impurities. In contrast, contemporary biochemistry and molecular biology make it possible to obtain pure allergen molecules. To date, 16 allergens of *A. alternata* have been isolated, many of which are enzymes: Alt a 4 (disulfide isomerase), Alt a 6 (enolase), Alt a 8 (mannitol dehydrogenase), Alt a 10 (alcohol dehydrogenase), Alt a 13 (glutathione-S-transferase), and Alt a MnSOD (Mn superoxide dismutase). Others have structural and regulatory functions: Alt a 5 and Alt a 12 comprise the structure of large ribosomal subunits and mediate translation, Alt a 3 is a molecular chaperone, Alt a 7 regulates transcription, Alt a NTF2 facilitates protein import into the nucleus, and Alt a TCTP acts like a cytokine. The function of four allergenic proteins, Alt a 1, Alt a 2, Alt a 9, and Alt a 70 kDa, remains unknown.

Keywords *Alternaria alternata* · Allergenicity · Fungal allergens · IgE · Recombinant allergens · Sensitizing proteins

Introduction

Alternaria alternata, the more common species of genus *Alternaria*, is a fungus whose spores occur worldwide in two main environments. Throughout the year, the spores can be found in the organic constituents of soil. From spring to autumn, they become airborne and are therefore even more ubiquitous. Though usually considered saprophytic contaminants, *Alternaria* is now held to be responsible for a number of disorders. Additionally, *Alternaria* is an important allergen, and the role of allergies to *Alternaria* in the development and exacerbation of asthma gains more and more recognition in medicine.

Biology of *A. alternata*

History of the Research

A. alternata first became known to the scientific community under the name *Alternaria tenuis*. In 1817, Christian Gottfried Daniel Nees von Esenbeck (1776–1858) established the genus *Alternaria* and described *A. tenuis* as its species (Fig. 1) [1, 2].

Having given rise to a taxonomy dispute that would continue long after his death, Nees went on to become a professor of botany and the director of the Botanical Garden in the prosperous German town Breslau [3]. Following the Yalta Conference in 1945, that German town would become known as the Polish city of Wrocław, where the authors of the present study work and reside. In 1851, Nees lost his professorship and the pension associated with it as a result of his political involvement; he died in poverty 7 years later.

I. Kustrzeba-Wójcicka · E. Siwak · G. Terlecki
Department of Medical Biochemistry, Wrocław Medical University,
Chafubińskiego10, 50-368 Wrocław, Poland

E. Siwak (✉) · A. Wolańczyk-Mędrala · W. Mędrala
Department of Internal Diseases, Geriatrics and Allergology,
Wrocław Medical University, Wrocław, Poland
e-mail: emiliasiwak88@gmail.com

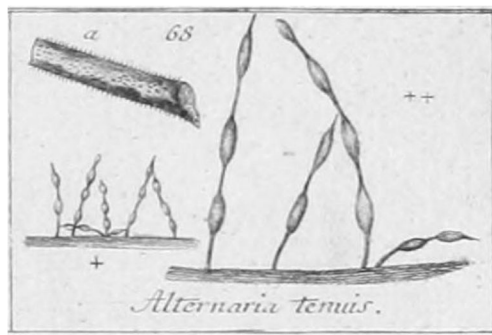


Fig. 1 Christian Gottfried Daniel Nees von Esenbeck's drawing of *A. tenuis*, the first known description of *Alternaria* (1817)

In 1912, Austrian mycologist and lichenologist Karl von Keißler (1872–1965), who continued the study of *A. tenuis*, renamed it *A. alternata*. In recognition of his research, the designation Keissl. was added to the name of the fungus [4].

Taxonomy

Kingdom: Fungi

Phylum: Ascomycota

Class: Dothideomycetes (Euascomycetes)

Order: Pleosporales

Family: Pleosporaceae

Genus: *Alternaria*

Species: *Alternaria alternata*

Morphology

A. alternata, which belongs to the family of black-pigmented molds that owe their hues to melanin, forms fast-growing colonies in dark colors ranging from gray to olive or olive brown. The surfaces of mature colonies may appear “fuzzy,” “downy to wooly,” or “suede like” [5] due to the presence of numerous hyphae. Microscopic observations show that the septate brown hyphae form conidiophores, which are also septate. Conidia, either single or forming chains, are simple and large, with the size $45\text{--}50 \times 36\text{ }\mu\text{m}$. It has been noted that, in natural habitats, the conidia are larger, are more uniform, and have elongated tips in comparison to those grown on agar media. The tips are shorter in conidia grown in cooler, dryer conditions [6]. The cells are surrounded by cell walls built of chitin and β -1,3-glucans. *A. alternata* produces dry, relatively large spores ($25\text{--}56 \times 8\text{--}17\text{ }\mu\text{m}$) which are “beak like,” elongated, and transversally septated [7]. The spores separate from the conidiophore in dry air both passively and under the influence of stronger gusts of wind. As Rotem notes, it is difficult to distinguish one species of *Alternaria* from others as “the microscopic differences between *Alternaria* species are not significant, and the character of every species varied depending on the conditions of growth.” The following measurements generally apply: maximum width of spore from 5–

13 to 6–24 nm, with a mean of 9 to 17 nm; length of spore from 16–37 to 35–110 nm with a mean of 37 to 69 nm; the number of transverse septa from 0 to 23; the beak length from 4 to 90 nm; and the conidiophore length from 20 to 300 nm [2].

Ecology

The lifestyle and ecological requirements of *A. alternata* have been determined. *A. alternata* fungi are classified as “outdoor” molds. They occur cosmopolitically in various environments, where they obtain nutrients in two ways. Saprophytic species can be found in plants, in the soil, and in foodstuffs. Parasitic species attack plants, animals, and humans, causing the development of alternariosis—a type of mycosis especially prevalent in immunocompromised patients. *A. alternata* is a mesohigrophile. A study conducted by Ren et al. showed that humid conditions of 84–89 and 97 % facilitated both growth of fungi and production of mycotoxins [11]. The spores require dry, warm, windy weather to become airborne and disseminate, so spore counts in air peak during sunny afternoons in late summer and early autumn and plunge to zero during the winter [8]. Although *A. alternata* will survive in temperatures from 2 to 32 °C, its temperature optimum is around 20 °C. Pose et al. studied the influence of temperature on the germination and growth of *A. alternata* on a synthetic medium. The shortest germination time was observed at 21–35 °C, while the fastest growth rate was registered at 21 °C. At 6–15 °C, no growth or germination was observed. A temperature of 6 °C caused a significant reduction in growth rates. For the synthesis of alternariol, 20 °C is the optimal temperature (Fig. 2), which is one of the main mycotoxins produced by *Alternaria* and can be found in food [9, 10].

A. alternata's predilection for higher temperatures means that it occurs more often in warm climates, i.e., the Mediterranean. In the subtropic and tropic zones, spore counts remain high throughout the year. In temperate climates, the spores mainly occur from May to November and the spore counts peak in late summer and autumn [12]. Kasprzyk et al. performed an interesting study of the occurrence of airborne *Alternaria* spores in three different regions of Poland:

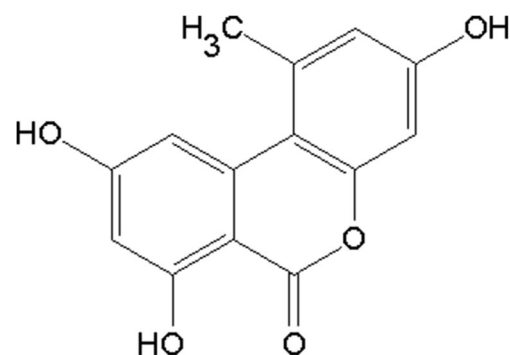


Fig. 2 Mycotoxin alternariol (3,7,9-trihydroxy-1-methyl-6H-benzo[c]-chromen-6-one)

Rzeszów, Lublin, and Poznań. The study was performed from 15 April to 30 September in two consecutive years. While acknowledging the influence of the climate on spore dissemination, the authors also indicate the importance of other factors in the prevalence of airborne spores in some regions. The landscape and geobotanical conditions of the area also play a role, together. The authors found the highest spore counts in Poznań, which is “the city with the highest urbanization factor and the capital of the region with large-scale farming, connected with the intensification of agricultural practices” [13].

Another Polish author, Krysińska-Traczyk, also finds the agricultural work environment to be highly polluted by fungal spores. In 8 of the 12 farms she studied, the air mold fungi concentration exceeded the standard limits. Especially high levels of *Alternaria* allergens were found to occur during grain threshing, flax breaking, and thyme cleaning [14]. Others also found higher levels of mold allergens in environments with high levels of bioaerosols such as poultry farms and sawmills [15].

While *Alternaria* is predominantly an outdoor allergen, exposure to *Alternaria* allergens can occur in the indoor environment. Studies performed by aerosampling showed that, when the outdoor spore counts reached 7,500 spores/m³ of air, the indoor spore counts could reach up to 280/m³ [8]. Within buildings, *Alternaria* is often to be found in textiles, such as carpets or bedding. A study focusing on the conditions in inner-city, low-income public housing developments, performed in 2002–2004, yielded the result that *Alternaria* antigen was found in all bed dust samples regardless of season [16]. The mold is also often found in water-damaged buildings or ones with a high humidity due to faulty air-conditioning or insufficient ventilation. In too-tightly-sealed windows, the humidity condenses on window panes and gathers on the flat surfaces of window frames, which makes these surfaces especially conducive to mold growth. The presence of *A. alternata* is one of the factors causing “sick building syndrome” [17].

Finally, a study by O'Connor et al. indicates that, alongside buildings with dampness problems, the houses that bear a greater risk of *A. alternata* allergen occurrence are those that have cockroach infestations or cats [18]. It may be assumed that the reason for higher concentrations of fungi in homes with cockroach infestations and cats is that the animals carry *Alternaria* spores on their bodies and transfer them into the house. Animal droppings also provide an environment for mold colonies. Their development is facilitated by high humidity which is often the result of defective isolation and outdated ventilation solutions associated with low-income communities [17, 19]. It would also seem that cockroach infestations and a large number of cats may be characteristic of homes where the hygienic standards are not excessively high. The concomitant lack of chemical cleaning agents is also conducive to the development of fungi.

Medical Significance

Pavon et al. provide a useful overview of the pathogenicity of *Alternaria*. They highlight the fact that cereals, vegetables, and fruit crops can be affected both before and during storage. While *Alternaria* is usually found in fresh fruit and vegetables, it can sometimes occur in preserves, juices, or sauces prepared from damaged products [20]. *Alternaria* species produce many metabolites toxic to plants, some of which are also toxic to animals and humans. However, when Edmondson et al. studied the “toxic mold syndrome,” they found allergic, rather than toxic, responses to be the main cause of symptoms in the studied group [21]. Simon-Nobbe et al., in turn, note that “in contrast to pollen, fungal spores and/or mycelial cells may not only cause type I allergy, the most prevalent disease caused by molds, but also a large number of other illnesses, including allergic bronchopulmonary mycoses, allergic sinusitis, hypersensitivity pneumonitis, and atopic dermatitis; and, again in contrast to pollen-derived allergies, fungal allergies are frequently linked with allergic asthma” [22].

The prevalence of *Alternaria* allergies in different geographic regions varies. A multicenter study conducted by D'Amato et al. analyzed data from allergic patients in a number of European countries. In a group of almost 900 patients with allergies, a hypersensitivity to *Alternaria* was found in 3–20 % of the group (3 % in Portugal to 20 % in Spain) [23]. In Poland, a different study of almost 500 adults with allergies found *Alternaria* to be the most frequent cause of allergic symptoms (47.1 %) [24]. For the Polish population, the threshold concentration of *Alternaria* spores, for which allergic reactions occur, is 80 spores/m³ of air. At this concentration, the clinical symptoms occur in the first sensitized patients. At 100 spores/m³, all the sensitized patients develop symptoms. At 150 spores/m³, the symptoms exacerbate and 300 spores/m³ dyspnea appears [25]. Figure 3 presents the concentration of spores in air in the Polish city of Wrocław in the course of 2 years: 2005 and 2010. As the graph shows, the concentration of spores in the air exceeded the limit of 80 spores/m³ almost throughout both seasons of *Alternaria* sporulation and peaked especially high in the beginning of August, when it exceeded the limit of 300 spores/m³, at which dyspnea

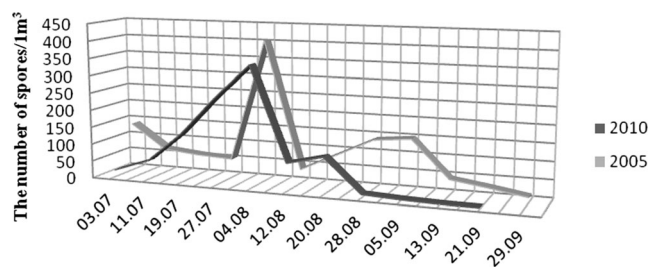


Fig. 3 A comparison of the concentrations of *A. alternata* spores in Wrocław in 2005 and 2010. The graph was created on the basis of data from [26, 27]

occurs. Thus, *Alternaria* spores may be considered an important allergen for the Wrocław population.

The *A. alternata* allergenic extracts have a great clinical relevance to the diagnosis of allergic diseases. They are a mixture of allergens of a given biological strength, causing a particular allergic reaction in the group of hypersensitive patients. It has been proved that proteases from *A. alternata* extracts cause the activation and degranulation of human eosinophils, one of the immune system components responsible for controlling mechanisms associated with the development of allergy and asthma [28]. In addition, eosinophil granulocytes are activated through the protease-activated receptor 2 (PAR-2), involved in the recognition of the antigens. Reed and Kita studied the stimulation of PAR receptors distributed in the airway epithelial cells with *Alternaria* extracts. They observed the phenomenon of opening of the tight junctions in the epithelium [29]. Furthermore, in response to this stimulation, human epithelial cells also produced biologically important cytokines and growth factors, including IL-6, IL-8, and also GM-CSF [30]. The mentioned substances are released during allergic reactions and play a proinflammatory role.

Of equal interest are the presented results of studies on mice carried out in 2014 by researchers from the USA. Kim et al. demonstrated that *Alternaria* extracts induce innate type 2 lung inflammation in mice [31]. Moreover, an extract of this mold may also enhance eosinophilic lung inflammation caused by ryegrass pollen, which represents the most potent source of allergens worldwide. The outcome of Kim's investigations shows that a single provocation trial with *Alternaria* extract enhances lung eosinophilia, peribronchial infiltration, and epithelial mucus production, which may lead to the development of severe asthma [31]. Medical reports also indicate that fungi are often involved in other allergic diseases such as chronic rhinosinusitis (CRS). Peripheral blood mononuclear cells from CRS patients after exposure to *Alternaria* produce IL-5, IL-13, and interferon- γ . It is also known that *Alternaria* induces IL-33 production and extracellular ATP release from normal human bronchial epithelial cells [32].

The current state of knowledge of *A. alternata* allergens does not allow to determine the medical significance of each described allergen in more detail. Experimental studies show that proteolytic activity of fungal extracts exhibits the ability to induce morphologic changes and cytokine production in airway epithelial cells. It must be assumed that the full allergen extracts, which are a mixture of proteins and other sensitizing chemical compounds of a given biological strength, cause an inflammatory reaction. It should therefore be assumed that multicomponent extracts of *Alternaria* allergens are predominantly involved in the pathogenesis of allergic diseases.

Nevertheless, in literature we can find the statement that for instance Alt a 1 is the main marker for evaluating sensitization to allergenic species belonging to the Pleosporaceae family

[33]. Furthermore, Wagner and other Austrian researchers demonstrated that the minor allergen- enolase (Alt a 6) may take part in the cross-reactivity phenomenon among several species that belong to different phyla [34]. It is also noteworthy that some *A. alternata* allergens, such as Alt a 3, Alt a 6, Alt a 10, and Alt a 13, may help to clarify diagnostic problems associated with poly-sensitization or cross-reactivity phenomenon [22].

A. alternata Allergens

History of the research

The first mention of an allergy caused by molds dates back to the very end of the seventeenth century. English physician Sir John Floyer noted the phenomenon of an adverse asthmatic reaction to mold as early as in 1698. His *A Treatise of the Asthma* describes the case of an "asthmatic who fell into a violent fit, by going into a wine cellar where the must was fermenting" [35–37]. It was not until the 1930s that allergic asthma was connected to sensitivity to *A. alternata*. Again, it was the cellar that was the culprit, as the patient's asthmatic symptoms were exacerbated after every visit to his basement. Hopkins et al. studied samples taken from the cellar and proclaimed it to be infected by *A. alternata* [38].

Studies on Allergen Composition

In regard to biochemistry, the development of allergology throughout the last century can be divided into three stages. The first stage encompassed research into the identification of raw materials causing allergies, such as house dust, pollen, or molds. The raw materials are still in use today as allergenic extracts. However, the usefulness of these extracts in diagnostics and therapy is limited because of their variability and insufficient standardization [22, 39]. In effect, as some note, allergenic extracts are still best described as "an unpredictable mixture of allergenic and non-allergenic compounds" [40]. This opinion is illustrated well by the results of an experiment carried out in the authors' laboratory, during which commercial mold fungi extracts used in allergological diagnostics were analyzed. The solutions for analysis were prepared according to the manufacturer's instructions. The concentrations of proteins and carbohydrates considered to be mold allergens were determined. The enzymatic activity of the panallergen enolase was also studied. The results of the experiment presented in Table 1 indicate a great biochemical variability of the extracts.

The observed variability of proteins and mannans, which are important allergens, was especially significant. The denaturation of enolase, which probably occurs during extract

Table 1 Biochemical analysis of mold extracts [43]

Allergen name	Protein concentration [mg/ml]	Concentration of sugars [mg/ml]	Enolase activity [IU]
<i>Alternaria alternata</i>	0.527	0.890	0.0
<i>Aspergillus fumigatus</i>	0.280	1.500	0.0
<i>Botrytis cinerea</i>	0.651	0.720	0.0
<i>Candida albicans</i>	0.095	0.333	0.0
<i>Curvularia lunata</i>	0.402	0.278	0.0
<i>Penicillium notatum</i>	0.173	0.283	0.0
<i>Helminthosporium halodes</i>	0.217	0.226	0.0
<i>Mucor mucedo</i>	0.132	0.263	0.0
<i>Neurospora sitophila</i>	0.534	0.975	0.0
<i>Pullularia pullulans</i>	0.362	1.870	0.0
<i>Fusarium moniliforme</i>	0.760	0.100	0.0
<i>Cladosporium herbarum</i>	1.159	0.435	0.0

preparation, makes it impossible to determine the enzymatic activity of the panallergen.

The second stage was research aimed at isolating and characterizing allergenic molecules obtained using classic biochemical methods. Native, purified allergens can be isolated from natural allergen sources. The authors' own research made it possible to create a method for purifying the panallergen enolase from *A. alternata*, *Cladosporium herbarum*, and *Candida albicans*. The physicochemical and kinetic properties of the purified enzymes were also identified. These preparation methods, however, require much labor while their effectiveness is limited, which makes them less useful in obtaining pure allergens in quantities sufficient for the purposes of diagnostics and therapy [41–43].

The third stage, finally, has been developing since the 1990s and deals with obtaining recombinant allergenic molecules (recombinant allergens). Using methods of molecular biology, mold allergens are obtained by cloning the genes encoding the allergens. These techniques lead to new diagnostic and therapeutic possibilities in allergology, such as molecular-based allergy diagnostics—that is, “a diagnostic approach to define the allergen sensitization of a patient at the molecular level using purified natural or recombinant allergen on singleplex or multiplex measurement platforms” [44]. In other words, molecular diagnosis “makes it possible to identify potential disease-eliciting molecules” [45]. The ImmunoCAP ISAC test is an example of the application of the recombinant allergens in immunodiagnostics. The ImmunoCAP ISAC Molds and other Microorganisms test contains, among others, the recombinant *A. alternata* major allergen—rAlt a 1 [46].

Allergen Nomenclature

Allergen nomenclature is the area of expertise of a Subcommittee of the World Health Organization and International

Union of Immunological Societies (WHO/IUIS). The current systematic allergen nomenclature is based on abbreviated Linnean genus and species names, followed by a space and an Arabic number for the order of allergen purification. For example, allergens that begin with “Alt a” are from *A. alternata*. In order to distinguish various allergens from the same species, a number is added to the name (e.g., Alt a 1, Alt a 2, etc.). The numbers are assigned to the allergens in the order of their identification. The system originated in 1980 and was refined twice, in 1986 and 1994. To be included in the WHO/IUIS nomenclature, the allergens must fulfill a set of criteria pertaining to their biochemical purity and allergenic activity. The biochemical criteria for including an allergen in the nomenclature state that the allergen must either be purified (using biochemical methods) or cloned (using the methods of genetic engineering). It is required that the full amino acid and/or nucleotide sequences be stated.

The allergenicity criteria for including an allergen in the nomenclature also state that the purified allergen must demonstrate allergenic activity in vitro and in vivo.

More specifically, an allergen will only be included if it is proven to induce IgE antibody production in at least five individuals. It is important to differentiate between major and minor allergens. It is generally held that a major allergen is one to which at least 50 % of allergic patients react [44, 47, 48].

Allergen Databases

There are a number of allergen databases available on the Internet. Of them all, two deserve special attention. The first is allergen.org [49], which is very restrictive in the criteria it uses as a basis for including an allergen. Conversely, the second one, allergome.org [50], aims to include as many of the known allergens as possible.

As the official website of the WHO/IUIS Sub-committee on Allergen Nomenclature, the database allergen.org lists all those allergens that have been recognized by that committee. As such, the allergens must fulfill the committee's restrictive criteria to be listed. The website is updated regularly [47, 48]. However, it has been noted that many well-characterized allergens are not in the WHO/IUIS list simply because they had not been submitted [40].

The allergome.org database seeks to address this problem. Its aim is to create a comprehensive list of allergens using the widest possible range of criteria. As Mari et al. note, the only requirement is that the allergen had been tested for its IgE-binding capacity at least once or that it has a structural relationship with other known allergens [40, 51].

Table 2 shows the hitherto recognized allergens of *A. alternata*.

The allergens listed on the WHO/IUIS website [49] are shown in blue (Alt a). The allergens that are listed on Allergome, a platform for allergen knowledge website [50], but not on the WHO/IUIS website [49], are shown in black (Alt a).

Molecular Characteristics of *A. alternata* Allergens

Alt a 1 Major Allergen

A major allergen of *A. alternata*, Alt a 1, was isolated using chromatographic methods. The obtained protein is a 29-kDa dimer with cysteine-linked subunits, which has an isoelectric point of about 4.2. It migrates as two separate 16.4- and 15.3-

kDa bands under reducing conditions on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [52]. For this Alt a 1 allergen, the sequence of 20 N-terminal amino acids was obtained and found to show a weak binding of patients' IgE [53]. The subunits of Alt a 1 were cloned; the expressed recombinant allergen, like the natural allergen, reacted with IgE from *A. alternata*-sensitive patients [54, 55]. This was later proved in a series of clinical studies: in a test of seven patients allergic to *A. alternata*, the whole studied group reacted to the recombinant Alt a 1 [56]. A different study analyzed specific IgE and IgG responses to rAlt a 1 in asthmatic and atopic dermatitis patients. Of the asthmatics and atopic dermatitis patients, 93 and 47 %, respectively, had antibodies to Alt a 1 [57]. When Asturias et al. tested *Alternaria*-allergic patients with natural and recombinant Alt a 1, 41 of the 42 patients specifically reacted with rAlt a 1 [58].

Recently, Chruszcz et al. used X-ray crystallography to obtain a high-resolution structure of Alt a 1 (Fig. 4). They found that Alt a 1 is a unique β -barrel which comprises 11 β -strands and forms a "butterfly like" dimer. The dimer is linked by a single disulfide bond with a large (1,345 Å²) dimer interface [59]. Twaroch et al. determined that Alt a 1 can be found in the cell walls of *A. alternata* spores [60]. The same authors undertook work on creating a safe, nonallergenic vaccine for specific immunotherapy (SIT) of *Alternaria* allergy. Peptides spanning regions of predicted high surface accessibility of Alt a 1 were synthesized and tested for IgE reactivity and allergenic activity, using sera and basophils from allergic patients. The study's results indicated carrier-bound nonallergenic Alt a 1 peptides as candidates for safe SIT of *Alternaria* allergy [61].

Despite of more than two decades of extensive and advanced studies on Alt a 1 described in this paragraph, the sequence of Alt a 1 remains recognized only in part, and its biological function remains unknown [63, 64].

Table 2 The allergens of *A. alternata*

Allergen	Biochemical name	MW (SDS-PAGE)
Alt a 1		16.4 and 15.3 band (30 nonred)
Alt a 2		
Alt a 3	Heat shock protein 70	
Alt a 4	Disulfide isomerase	57
Alt a 5	Ribosomal protein P2	11
Alt a 6	Enolase	45
Alt a 7	YCP4 protein	22
Alt a 8	Mannitol dehydrogenase	29
Alt a 9		
Alt a 10	Aldehyde dehydrogenase	53
Alt a 12	Acid ribosomal protein P1	11
Alt a 13	Glutathione-S-transferase	26
Alt a 70 kDa		
Alt a MnSOD	Mn superoxide dismutases	
Alt a NTF2	Nuclear transport factors	
Alt a TCTP	Translationally controlled tumor proteins	



Fig. 4 Crystal structure of the *A. alternata* major allergen Alt a 1 [62]

Alt a 2

The Alt a 2 allergen was cloned by Bush et al. and found to be a major allergen of *A. alternata*; it was presumed to bind IgE from 61 % of patients sensitive to *Alternaria* [65]. However, a study by Asturias et al. found much different results: none of the 42 patients in that study reacted with Alt a 2, which leads to the conclusion that Alt a 2 was in fact a minor, instead of a major, allergen [58]. Currently, the 25-kDa [22, 66] complete allergen sequence of 190 amino acids is recognized, while its biochemical function remains unknown [64].

Alt a 3

Heat Shock Protein 70 kDa

Another step in identifying *A. alternata* allergens was the cloning of Alt a 3. De Vouge et al. cloned the sequences that encode IgE-binding fragments of an allergen from *A. alternata*. The fragments were homologous to a region of heat shock protein 70 kDa (HSP-70) from another mold, *C. herbarum*. Alt a 3 was recognized on immunoblot by 5 % of the sera from *A. alternata*-sensitive patients [55]. The sequence of Alt a 3 is recognized only partially (152 amino acids) [64]. Its biochemical function as HSP-70 is that of a molecular chaperone which protects cells from thermal and oxidative stress [67].

Alt a 4–7, Alt a 10, and Alt a 12

In the middle of the 1990s, Achatz et al. cloned Alt a 4–7, Alt a 10, and Alt a 12. The cloned allergens were sequenced and expressed as recombinant proteins. On the basis of their very high homology with known sequences, their biological functions were deduced [68, 69]. Detailed information on the structures and biochemical functions of these allergens are presented below.

Alt a 4

Alt a 4 is a 57-kDa enzyme—protein disulfide isomerase—that binds IgE from the sera of 42 % of *Alternaria*-sensitive individuals [22, 66, 68, 69]. The molecular structure of Alt a 4 was also analyzed. Even though its sequence is incomplete (436 amino acids), it is known that an Alt a 4 molecule contains at least one thioredoxin domain [60]. The biochemical “household” function of protein disulfide isomerase (PDI) EC 5.3.4.1. is that it catalyzes disulfide oxidation, reduction, and isomerization, thereby playing an important role in protein synthesis as well as in forming the tertiary structure of proteins in the endoplasmic reticulum of eukaryotic cells [70].

Alt a 5 (Obsolete Name Alt a 6)

Alt a 5 is a 11-kDa 60S acidic ribosomal protein P2 and binds IgE from the sera of 8–14 % of *Alternaria*-sensitive patients [22]. The allergen is a phosphorylated ribonucleoprotein whose sequence is complete (113 amino acids) [64]. The biochemical function of 60S acidic ribosomal protein P2 is well known: it is a structural constituent of the large ribosomal subunit directly involved in the elongation step during the course of protein synthesis [71].

Alt a 6 (Obsolete Name Alt a 5, Alt a 11)

Alt a 6 is an enolase, also called phosphopyruvate hydratase or 2-phospho-D-glycerate hydrolyase EC 4.2.1.11. It was first described in 1934 by Lohman and Meyerhof as an enzyme catalyzing the reversible conversion of 2-phospho-D-glycerate (2PGA) into phosphopyruvate (PEP) [72]. Initially, enolase was known as a cytosolic enzyme cooperating in the most important glucose metabolism pathways—glycolysis and gluconeogenesis. Later studies have shown that it is a multifunctional protein that can occur in various cell compartments [73, 74]. It was first identified as an allergen in *Saccharomyces cerevisiae* by Baldo and Baker [75]. In the authors’ own studies, 95 % of patients sensitized to *C. albicans* extract showed positive skin reactions to *S. cerevisiae* enolase [76]. Currently, enolase is considered to be a mold fungi panallergen.

The enolase from *A. alternata* was obtained independently in two separate research centers. Austrian scientists cloned Alt a 6 and found that 22 % of the *A. alternata*-allergic patients specifically reacted to the enzyme [22, 68, 77, 78]. The molecular weight of the recombinant enolase from *A. alternata* was 45 kDa. In the authors’ research team, the purification of enolase from *A. alternata* was achieved by anion-exchange chromatography and size-exclusion chromatography after ammonium sulfate protein precipitation from crude extracts. The highly purified, electrophoretically homogenous enzyme has a subunit molecular weight of 47 kDa and a pH optimum of 6.8. The kinetic parameters for the 2PGA→PEP reaction were determined: $K_M=0.36$ mM and $V_{max}=4$ mmol min⁻¹ μmol⁻¹. The enolase was shown to possess a high thermostability, allowing for 50 % of activity to remain after 1 h of incubation in 50 °C [42]. Properties of the enzyme from *A. alternata* are close to those of enolases from *C. albicans* and *C. herbarum* [41, 43].

In our in silico studies of the molecular evolution of enolase, we have confirmed that enolase is found from bacteria to mammals, and its sequence is highly conserved throughout evolution [79].

Alt a 7

Alt a 7 is a 22-kDa homologue of the yeast protein YCP4 and binds IgE in 7 % of the *Alternaria*-sensitive people [22, 66,

[68]. The complete 204-amino acid sequence of Alt a 7 is known, while its function remains unidentified [64]. YCP4 is similar in sequence and structure to flavodoxins—proteins that have been detected in highly purified mitochondria [80]. Flavodoxins contain FMN and function as electron transfer agents in a variety of microbial metabolic processes [81, 82]. Lately, it has been found that flavodoxin-like YCP4 acts as a transcriptional regulator [83].

Alt a 8

Alt a 8 is a 29-kDa enzyme-NADP-dependent mannitol 2-dehydrogenase (MtDH) EC 1.1.1.138 cloned by Schneider et al. In IgE-ELISA and immunoblots, recombinant Alt a 8 was recognized by 41 % of *A. alternata*-allergic patients. In vivo immunoreactivity of the allergen was verified by skin prick testing [22, 84]. The complete 266-amino acid sequence of Alt a 8 is known [60]. The kinetic parameters of MtDH have been determined: $K_M=474$ mM for D-fructose and $K_M=18.7$ μ M for NADPH [84]. The biochemical function of MtDH is that it takes part in both mannitol synthesis and degradation by catalyzing the reversible conversion of mannitol to fructose. Mannitol is the most common polyol of fungi; it can be found in high concentration in mycelia and spores. It plays different roles in fungi: it serves as a storage carbohydrate or translocated carbohydrate and is necessary in the process of pathogenesis. However, mannitol is not required for spore germination or initial infection [85, 86].

Alt a 9

Alt a 9 is a protein of unknown structure and biochemical function. Some authors report that its molecular weight is 43 kDa and that 5 % of patients are hypersensitive to this allergen [68, 87]. Alt a 9 is listed in the Allergome database, but not in the official database of the WHO/IUIS Subcommittee on Allergen Nomenclature.

Alt a 10

Alt a 10 is a 53-kDa NAD-dependent aldehyde dehydrogenase (ALDH) EC 1.2.1.3 that binds IgE in 2 % of sensitive patients [68]. Its complete 497-amino acid sequence is known [64]. Its biochemical function is cooperation in ethanol catabolism. ALDH metabolizes ethanol-derived acetaldehyde and has a broad substrate specificity which includes a variety of aldehyde structures [88].

Alt a 12

Alt a 12 is an 11-kDa 60S acidic ribosomal protein P2. Similarly to Alt a 5, which is the 60S acidic ribosomal

protein P1, the allergen is a phosphorylated ribonucleoprotein whose sequence is complete (110 amino acids). P1 and P2 form dimers that play a structural role in large ribosomal subunits and take part directly in the process of protein synthesis [68].

Alt a 13 Major Allergen

Alt a 13 is a 26-kDa glutathione-S-transferase (GST) EC 2.5.1.18 that reacted with sera IgE from 82 % of *Alternaria*-hypersensitive patients [89].

Recombinant rGST and native nGST from *A. alternata* are similar in their allergenicity and enzymatic activity [89]. GST was recognized as a cross-reactive allergen in fungal extracts [90]. IgE-binding fragments of Alt a 13 were determined by in silico studies and then cloned [91].

The complete 231-amino acid sequence of Alt a 13 is known [64]. The enzyme belongs to the GST superfamily, which consists of multifunctional cytosolic and membrane-associated microsomal proteins. The biochemical function of GST is biotransformation (detoxification) of endogenous and xenobiotic compounds by conjugation of these compounds to reduced glutathione. Glutathione conjugates are usually less toxic than the parent compounds. Because they are also more polar, they can be stored in vacuoles or excreted outside of the cell [92].

Alt a 70 kDa

Alt a 70 kDa is a protein of unknown structure and biochemical function [64]. Portnoy et al. report that it is a glycoprotein which forms 13 % of the dry weight of *Alternaria* extracts and causes positive skin test results in 87 % (14/16) of those *Alternaria*-sensitive patients [93].

Alt a MnSOD (Mn Superoxide Dismutase)

Alt a MnSOD is a mitochondrial enzyme, Mn-dependent superoxide dismutase EC 1.15.1.1.

Postigo et al. found that 6.6 % of *Alternaria* allergies can be ascribed to reactions to MnSOD without Alt a 1 sensitization. The authors also propose that, alongside Alt a 1 and enolase, MnSOD should be included in the molecular array for the diagnosis of allergy to Pleosporaceae [33, 94]. Only a 25-amino acid fragment of Alt a MnSOD molecular sequence is known. Its biochemical function, on the other hand, is clear. MnSOD is an antioxidant enzyme that catalyzes the dismutation of highly reactive superoxide anion radicals to hydrogen peroxide and oxygen. Hydrogen peroxide is subsequently converted by the catalase and glutathione peroxidase to water and oxygen.

Alt a NTF2 (Nuclear Transport Factor 2)

Alt a NTF2 is considered to be a putative NTF2 [95]. Weichel et al. obtained proteins, consisting of 124 and 125 amino acids, which corresponded to NTF2. The recombinant proteins were able to bind and cross-inhibit IgE binding and to elicit type I skin reactions in mold-sensitized individuals [95]. NTF2, a 28-kDa small homodimeric protein, is a cytosolic factor for protein import into the nucleus through the nuclear pore complex [96].

Alt a TCTP (Translationally Controlled Tumor Protein)

TCTP is also known as histamine-releasing factor HRF and fortilin [97–99]. Rid et al. cloned and studied the *A. alternata* homologue of TCTP and found that the recombinant *A. alternata* TCTP showed a roughly 4 % prevalence of IgE reactivity and that it has similar cross-reactive IgE epitopes as its *C. herbarum* homologue [100, 101]. TCTP is a highly conserved, multifunctional protein present in all eukaryotic organisms. TCTP evinces growth-promoting and antiapoptotic properties as well as cytokine-like activity [102]. TCTP takes part in cell cycle progression, proliferation, survival, and malignant transformation of cells [99]. Its expression is active in mitotically active tissues as a conserved mitotic growth integrator [103]. In its cytokine-like activity, TCTP is a proinflammatory factor and cooperates in the allergic process in higher organisms. TCTP is secreted by macrophages and other cells; it then stimulates histamine release and interleukin production from IgE-sensitized basophils and mast cells [97, 104]. In their latest studies, Kim et al. suggest dimerization as the critical, if not the sole, modification of TCTP that causes it to act in a cytokine-like manner and initiate allergic diseases [105].

Conclusions

There are currently 16 known allergens of *A. alternata*, ten of them are listed in the official database of the WHO/IUIS Subcommittee on Allergen Nomenclature. From the middle of the 1990s onwards, a team of scientists from the Salzburg University in Austria performed a series of experimental studies resulting in cloning a number of the allergens of *A. alternata* (Alt a 4–12, Alt a NTF2, Alt a TCTP). Over the next decade, the scientists from that team significantly enhanced the knowledge about the allergens of the mold. In silico analyses performed by Bowyer et al. complement the extensive experimental studies in molecular allergology. The authors studied 22 fungal genomes, including the genome of *A. alternata*, and identified the conserved allergen orthologues and the genes that code unique allergens. The conserved group

of allergens include, among others, enolase, heat shock proteins, thioredoxins, Mn superoxide dismutase, and disulfide isomerases. Allergens that occur uniquely in each genus of fungi form a separate group, containing, among others, Alt a 1 and Alt a 2 [106].

Several studies have found that there is a noticeable correlation between the severity of allergic diseases and the proteolytic activity of *Alternaria* extracts. The activation of airway epithelial cells with fungus *Alternaria* enhances lung eosinophilia, peribronchial infiltration, and epithelial mucus production, which are typical symptoms in asthma [28].

Fungal hypersensitivity is still not as well defined as other allergies, like pollen, mites, foodstuffs, insects, and animal dander hypersensitivity. Currently, the diagnosis of allergic diseases is based on the performance of skin prick tests and determination of specific immunoglobulin E (sIgE), circulating in the serum of patient. Unfortunately, due to the lack of fungal extracts standardization, skin testing with commercial mold extracts often shows poor specificity and sensitivity [56, 58, 107]. It is for this reason that research into the structure and properties of mold allergens should be continued, as new knowledge in this area would make it possible to improve the biochemical quality of the tests and vaccines widely available in the pharmaceutical industry.

Allergen-specific immunotherapy, as a method for treating the causes IgE-mediated allergies, promises to become a valuable solution for contemporary allergy treatment. Desensitization of a patient typically relies on the administration of increasing allergen concentrations or allergen mixtures by subcutaneous injection. It should be noted, however, that specific immunotherapy with commercial allergens extracts bears the risk of serious side effects, such as anaphylactic reaction [108]. The use of recombinant allergens, obtained by genetic engineering, for vaccine production makes it possible to minimize this risk as it eliminates the impurities present in traditional extracts. In clinical practice, this would require a precise tailoring of a vaccine's composition to the needs of a given patient, which would improve the effectiveness of immunotherapy individualization. Due to this fact, future diagnosis and desensitization of mold-allergic patients is a great challenge for current molecular medicine and biotechnology.

Conflict of Interest Irena Kustrzeba-Wójcicka, Emilia Siwak, Grzegorz Terlecki, Anna Wolańczyk-Mędrala, and Wojciech Mędrala declare that they have no conflict of interest.

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